Kidney International, Vol. 57 (2000), pp. 2043–2054

ION CHANNELS – MEMBRANE TRANSPORT – INTEGRATIVE PHYSIOLOGY

Transport of thiamin in rat renal brush border membrane vesicles

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Transport of thiamin in rat renal brush border membrane vesicles.

Background. Unlike intestinal absorption, renal transport of thiamin has received little attention. This study was designed to investigate the reabsorptive steps of thiamin transport in brush border membrane vesicles (BBMVs) from rat kidney proximal tubules using tritiated thiamin with a high specific activity.

Methods. BBMVs prepared from the cortex kidney of rats were suspended in different media, controlling the composition of the intravesicular fluid by prolonged equilibration at 4°C in preincubation buffers of desired composition. Then they were held on ice until used, when they were warmed at 25°C for the uptake experiments. The amount of radioactivity taken up by the vesicles was measured radiometrically after separation with a rapid-filtration procedure.

Results. The time course profile of thiamin uptake was Na^+ independent; 53% of thiamin taken up was membrane bound. The concentration curve had a biphasic course that was nonlinear (saturable) at physiological concentrations (<1.25 μmol/L) and linear at higher ones. Thiamin uptake was stimulated several-fold by an outwardly directed H^+ gradient (pH_{in} 6:pH_{out} 7.5), which caused a transient accumulation of thiamin inside BBMVs against a concentration gradient. The enhanced thiamin uptake was only due to the H^+ gradient, which made thiamin binding virtually negligible compared with translocation, and maintained the biphasic course of the concentration curve. The saturable component, however, had kinetic constants 12-fold higher than those in the absence of gradient. Moreover, the saturable component was inhibited by nonlabeled thiamin and its structural analogues, by inhibitors of intestinal thiamin/H^+, renal guanidine/H^+, and Na^+/H^+ antiporters, while it remained unmodified by some typical organic cations transported in renal BBMVs.

Conclusion. The results provide strong evidence for the presence in renal BBMVs of a thiamin/H^+ antiport having a 1:1 stoichiometric ratio.

Key words: renal transport, BBMV, thiamin/H^+ antiport, proximal tubules, vitamin.

Received for publication April 1, 1999
and in revised form October 18, 1999
Accepted for publication December 10, 1999

Thiamin is required by animal cells for the synthesis of thiaminpyrophosphate, the coenzyme of the important carbohydrate enzymes transketolase, pyruvate-, and α-ketoglutarate dehydrogenases [1]. The thiamin plasma concentration is regulated by both intestinal and renal mechanisms.

Transport of thiamin in the small intestine has received considerable attention. In all animal species, including humans, low (physiological) concentrations of thiamin are absorbed in vivo by a saturable transport mechanism [2, 3]. Thiamin entry into the enterocyte, as evaluated in rat brush border membrane vesicles (BBMVs), is a Na^+-independent process, mainly involving a saturable component at concentrations of less than 1.25 μmol/L and a nonsaturable component at higher concentrations. In addition, thiamin entry is inhibited by thiamin structural analogues and derivatives [4], and involves the countertransport thiamin/H^+ [5]. The exit of thiamin from the enterocyte, as evaluated in basolateral membrane vesicles, is Na^+-dependent, being directly coupled to ATP hydrolysis by Na^+,K^+-ATPase, and hence to the functional integrity of this enzyme, and is significantly inhibited by thiamin structural analogues [6]. In intestinal tissue, thiamin transport has been shown to be associated with its phosphorylation to thiaminpyrophosphate by thiamin pyrophosphokinase (EC 2.7.6.2.) [7].

Chemically, thiamin is an organic cation and precisely a quaternary ammonium compound. Its plasma levels are regulated, in part, by the kidney like those of other organic cations. At high plasma levels, these cations are actively secreted [8], whereas at low plasma levels, they are actively reabsorbed [9, 10]. Such a mechanism has also been demonstrated for thiamin in humans by injecting different doses of vitamin intravenously [11].

The thiamin level in rat plasma typically does not exceed 240 nmol/L [12, 13] and is not protein bound [14–16]. Therefore, under normal circumstances, thiamin is reabsorbed, rather than secreted, by the proximal tu-

bule [11]. Despite this fact, the reabsorptive steps for thiamin have attracted little attention [17].

In the present investigation, we studied the transport features for thiamin in BBMVs from rat kidney proximal tubules using tritiated thiamin ([^{3}H]-thiamin) with a high specific activity. First, we evaluated the general properties of thiamin uptake (time course profile, influence of Na^+ and K^+, concentration curve, and membrane binding). Then we focused our attention on the peculiarities of transport of thiamin as an organic cation by studying the influence of externally imposed membrane negative or positive potential, the effect of H^+ gradients differently oriented, the stoichiometry of thiamin/H^+ antiport, and the molecular specificity of the organic cations transported by the saturable component of thiamin/H^+ antiport.

METHODS

Animals

Adult Wistar albino rats (350 to 400 g body wt) of either sex, reared on a complete standard diet containing 12 μg/g thiamin [18], were used. Animals were killed by decapitation after 12 hours of fasting with water ad libitum.

Preparation of brush border membrane vesicles

Brush border membrane vesicles were prepared with minor modifications from the kidney cortex of five to six rats according to the method of Biber et al [19]. Cortex slices were homogenized for three minutes in an Ultra-Turrax homogenizer (T-25; Janke und Kunkel IKA-Labortechnik, Staufen-im-Breisgau, Germany), and all centrifugation steps were performed at 0 to 4°C in a Sorvall RC-5 centrifuge using a SS-34 rotor (Du Pont Instruments, Newton, CT, USA).

The vesicles were washed with and suspended in different media according to the type of the experiment. The composition of the intravesicular fluid of BBMVs was controlled by prolonged (at least 2 h) equilibration at 4°C in a preincubation buffer of desired composition (discussed in all of the figure legends). BBMVs were held on ice until they were used and were warmed at 25°C in a thermostatic bath for 30 minutes before the beginning of uptake experiments. The purity of microvillous membranes was estimated by assessing the enrichment in alkaline phosphatase and K^+-phosphatase activities of BBMVs, determined according to Murer et al [20], compared with the initial kidney cortex homogenate. The enrichments were 15.5 ± 0.8 times (mean ± SEM of 5 different preparations) for alkaline phosphatase and 1.6 ± 0.3 times (mean ± SEM of 5 different preparations) for K^+-phosphatase.

Protein content was determined according to Lowry et al [21], using bovine serum albumin as the standard. Spectrophotometric measurements were carried out with an Uvikon 930 Spectrophotometer (Kontron Instruments, Milan, Italy).

Transport efficiency of brush border membrane vesicles

The transport efficiency of the vesicular preparations was evaluated by determining the time course profile of D-glucose uptake by BBMVs. These were incubated with 80 μmol/L [U-^{14}C]-D-glucose (specific activity, 0.31 GBq/mmol) in the presence of an initial gradient (100 mmol/L out) of NaCl or KCl, as described by Casirola et al [4].

Thiamin uptake measurements

Brush border membrane vesicles were incubated at 25°C with [^{3}H]-thiamin (specific activity, 27.75 GBq/mmol). This temperature was chosen to reduce thiamin uptake rate so that early uptake events could be followed more accurately. Typically, a 90 μL aliquot of transport medium, containing appropriate concentrations of [^{3}H]-thiamin and salts (discussed in the figure legends), was rapidly mixed with 10 μL of vesicle suspension (0.1 to 0.2 mg protein). After terminating the incubation with 3 mL of cold (0 to 4°C) stopping solution (150 mmol/L NaCl and 1 mmol/L Tris-HEPES, pH 7.5), the amount of thiamin radioactivity taken up by the vesicles was measured radiometrically after their separation by the rapid filtration procedure of Kessler et al [22] using cellulose nitrate microfilters (pore diameter 0.65 μm; Microfiltration System, Dublin, CA, USA), previously saturated with unlabeled thiamin [4]. Blanks were prepared in each experiment to evaluate the radioactivity of [^{3}H]-thiamin nonspecifically adsorbed on the microfilter. The values of the blanks were subtracted from the total radioactivity retained on the filter. Radiometric measurements were carried out using a Packard Tri-Carb 2000 CA liquid scintillation counter (Packard Instruments CO, Inc., Downers Grove, IL, USA). Unless stated otherwise, all uptake values were means ± SEM of at least triplicate determinations for each of five different preparations.

For incubation times ranging from 1 to 15 seconds, a STRUMA short-time incubation apparatus (Innovativ-Labor, Adliswil, Switzerland) was used.

Statistics

The significance of the differences of the means under different experimental conditions was evaluated by using the analysis of variance (ANOVA), followed by Newman-Keuls's *Q*-test, and Student's *t*-test for paired data. All statistical tests were carried out with a computerized program [23].

Reagents

Unlabeled thiamin chloride hydrochloride and thiocrome were obtained from Prodotti Roche (Milan, Italy). Pyrithiamin bromide hydrobromide and 4′-oxythiamin

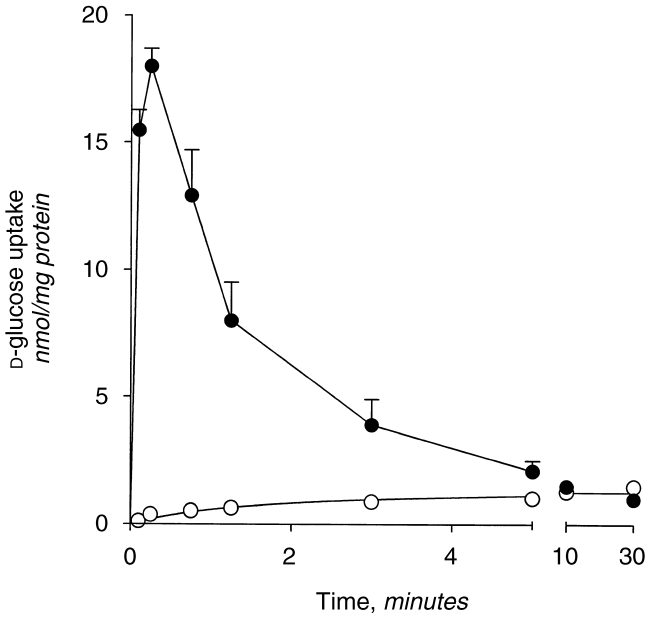

Fig. 1. Time course of D-glucose uptake by rat renal brush border membrane vesicles. Vesicles were preincubated (2 h at 4°C and 30 min at 25°C) in a medium containing (mmol/L) 280 D-mannitol, 2 $MgSO_4$, 10 Tris-HEPES, pH 7.5. Ten microliters of preincubated vesicles were incubated at 25°C with 90 μL of a solution containing 80 μmol/L [U-^{14}C]-D-glucose (specific activity, 0.31 GBq/mmol), 100 mmol/L D-mannitol, 2 mmol/L $MgSO_4$, 10 mmol/L Tris-HEPES, pH 7.5, and 100 mmol/L NaCl (●), or 100 mmol/L KCl (○). The symbols represent means ± SEM of triplicate determinations for each of five different vesicles preparations, each from five to six rats.

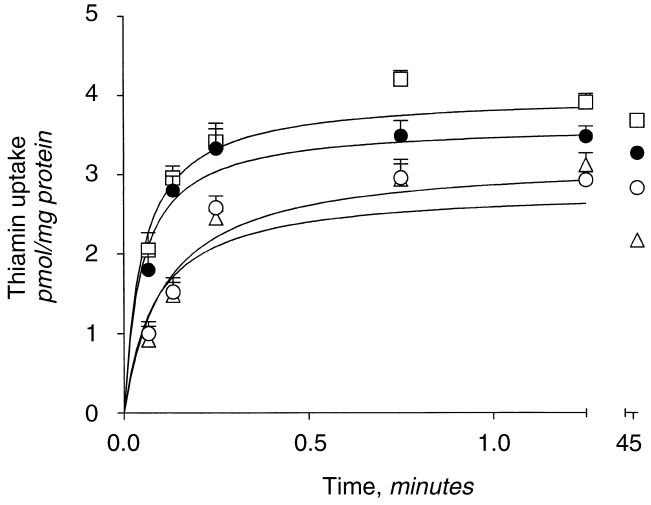

Fig. 2. Time course of thiamin uptake by rat renal brush border membrane vesicles. Vesicles were preincubated as described in Figure 1 in media containing (mmol/L) 2 $MgSO_4$, 10 Tris-HEPES, pH 7.5, and 300 D-mannitol (open symbols), or 100 NaCl and 100 D-mannitol (solid symbols). Ten microliters of preincubated vesicles were incubated at 25°C with 90 μL of media containing 0.25 μmol/L [^{3}H]-thiamin (specific activity, 27.75 GBq/mmol), 100 mmol/L D-mannitol, 2 mmol/L $MgSO_4$, 10 mmol/L Tris-HEPES, pH 7.5, 100 mmol/L NaCl [(○) out; (●) in = out], 100 mmol/L KCl (△), or 200 mmol/L D-mannitol (□). The number of experiments for each symbol is the same as in Figure 1.

chloride were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Amprolium was from Merck, Sharp, and Dohme (Pavia, Italy). EGTA [ethylenebis (oxyethylene-nitrilo) tetraacetic acid], MES hydrate (β-morpholine-ethane sulfonic acid), and FCCP [carbonylcyanide p-(trifluoromethoxy) phenylhydrazone] were from Aldrich Chimica (Milan, Italy). All other reagents were of analytical grade and were supplied by Sigma Chimica (Milan, Italy) and BDH Ltd. (Poole, Dorset, UK).

Labeled compounds

[U-^{14}C]-D-glucose (specific activity, 10.8 GBq/mmol) and [^{3}H]-thiamin (specific activity, 429.2 GBq/mmol) were from Amersham International PLC (Amersham, UK).

RESULTS

Transport efficiency of brush border membrane vesicles

The time course profile of D-glucose in the presence of an initial inwardly directed 100 mmol/L NaCl gradient (out) showed an overshoot between 6 and 45 seconds, which disappeared in the presence of 100 mmol/L KCl. In both inwardly directed gradient conditions, equilibrium was reached at 10 to 30 minutes (Fig. 1). This time course was similar to that reported in the literature [24], indicating that the preparation was suitable for transport studies.

Time course and Na^+ effect

Brush border membrane vesicles were incubated at 25°C with 0.25 μmol/L [^{3}H]-thiamin under four different conditions: (*1*) in the presence of an initial gradient 100 mmol/L NaCl out; (*2*) in 100 mmol/L KCl out in the incubation medium; (*3*) in the absence of alkaline ions, osmotically substituted with D-mannitol; and (*4*) in the presence of 100 mmol/L NaCl at both sides of the membrane vesicles, obtained by using BBMVs resuspended in a medium containing 100 mmol/L NaCl.

The time course of thiamin uptake remained virtually unchanged under all four conditions (Fig. 2).

Translocation and binding

To differentiate between translocation of thiamin into the intravesicular space and membrane binding, BBMVs were incubated with 0.25 μmol/L [^{3}H]-thiamin dissolved in media with increasing and osmometrically controlled (Fiske OM osmometer; Fiske Associates, Burlington, MA, USA) osmolarities ranging from 330 to 929 mOsm/L. When the values of equilibrium vesicular uptake of thiamin (30 min) were plotted against the reciprocal of medium osmolarities, a straight line was obtained. At infinite osmolarity (corresponding to the *y* intercept), only uptake by binding remains because vesicle volume tends

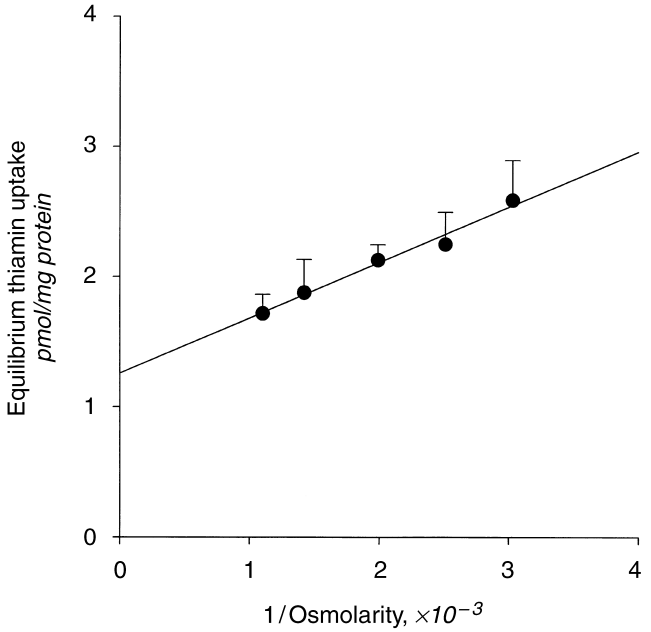

Fig. 3. Effect of medium osmolarity on thiamin uptake by rat renal brush border membrane vesicles. Vesicles were preincubated as described in Figure 1 in different media containing (mmol/L) 96, 164, 272, 470 or 695 D-mannitol, 2 $MgSO_4$, 90 NaCl and 25 Tris-HEPES, pH 7.5, and were incubated for 30 minutes at 25°C in media containing 0.25 μmol/L [^{3}H]-thiamin, 2 mmol/L $MgSO_4$, 90 mmol/L NaCl, 25 mmol/L Tris-HEPES, pH 7.5, and D-mannitol to yield the indicated osmolarities (mOsm/L; given as its reciprocal). The fitting was calculated by regression analysis ($r = 0.9905$, $P \leq 0.001$). The number of experiments for each symbol is the same as in Figure 1.

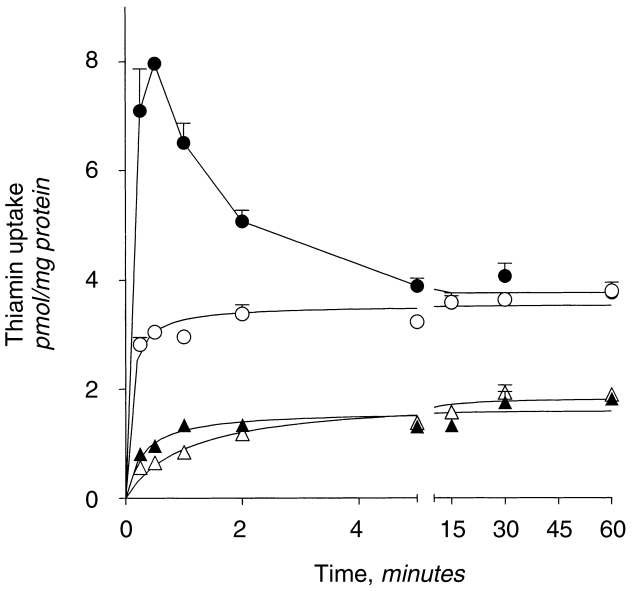

Fig. 4. Effect of pH gradients between 6 and 7.5 on the time course of thiamin uptake by rat renal brush border membrane vesicles. Vesicles were preincubated in media containing (mmol/L) 250 D-mannitol, 2 $MgSO_4$, 50 Tris-HEPES, pH 7.5 (open symbols), or Mes-Tris, pH 6 (solid symbols). Ten microliters of preincubated vesicles were incubated at 25°C with 90 μL of a solution containing 0.25 μmol/L [^{3}H]-thiamin, 250 mmol/L D-mannitol, 2 mmol/L $MgSO_4$, 50 mmol/L Mes-Tris, pH 6 (triangles), or 50 mmol/L Tris-HEPES, pH 7.5 (circles). The number of experiments for each symbol is the same as in Figure 1.

to zero. Moreover, the intercept was 0.53, indicating that binding was responsible for 53% of uptake (Fig. 3).

H^+ gradient and time course

After pre-equilibration, BBMVs were incubated with 0.25 μmol/L [^{3}H]-thiamin in the presence or in the absence of differently oriented H^+ gradients (pH_{in} 6:pH_{out} 7.5 or vice versa). The rate of thiamin uptake was influenced by H^+ gradient orientation, being greatly enhanced by the outwardly directed gradient ($pH_{in} < pH_{out}$; Fig. 4). At 30 seconds, the amount of thiamin taken up showed an overshoot that was about 2.5 times as large as the uptake observed in the absence of H^+ gradient ($pH_{in} = pH_{out} = 7.5$). This difference disappeared at equilibrium (15 to 60 min). The inwardly oriented H^+ gradient (pH_{in} 7.5:pH_{out} 6) or the absence of gradient in the acid side of pH ($pH_{in} = pH_{out} = 6$) caused a further significant reduction (92 and 88%, respectively) of thiamin uptake at 30 seconds, possibly as a consequence of competitive inhibition between H^+ and thiamin at the external face of the exchanger [25]. A remarkable confirmation of the importance of the outwardly directed H^+ gradient in stimulating thiamin uptake came from the use of the protonophore carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP), which promotes H^+ transport [26], thus dissipating H^+ gradient. At pH gradient pH_{in} 6:pH_{out} 7.5, the presence of 60 μmol/L FCCP in the preincubation medium resulted in an approximately 75% decrease of thiamin uptake at 15 seconds, which was associated with the elimination of the overshoot (Fig. 5).

Transmembrane electrical potential

To differentiate between the effect of the pH gradient and that of related changes in transmembrane electrical potential on thiamin transport, an electrical negative (or positive) potential was imposed across the BBMVs. For this purpose, the uptake of 0.25 μmol/L [^{3}H]-thiamin was measured at pH 7.5 ($pH_{in} = pH_{out}$) in the presence of K^+ salts of inorganic anions showing different permeabilities across the brush-border membrane. Incubation with the more liposoluble I^- (about 20-fold as compared with Cl^- and K^+) [25], created a relatively more negative or positive intravesicular compartment, depending on whether the anion was present outside or inside the vesicles. No differences in [^{3}H]-thiamin uptake were found under these conditions, except a significant increase at the 15-second incubation in the presence of an inside-positive electrical potential (Fig. 6). A similar stimulatory effect was found by Wright and Wunz, using the typical H^+-exchangeable organic cation tetraethylammonium [25].

The null effect of an inside negative potential on thiamin transport was confirmed by inducing a negative intra-

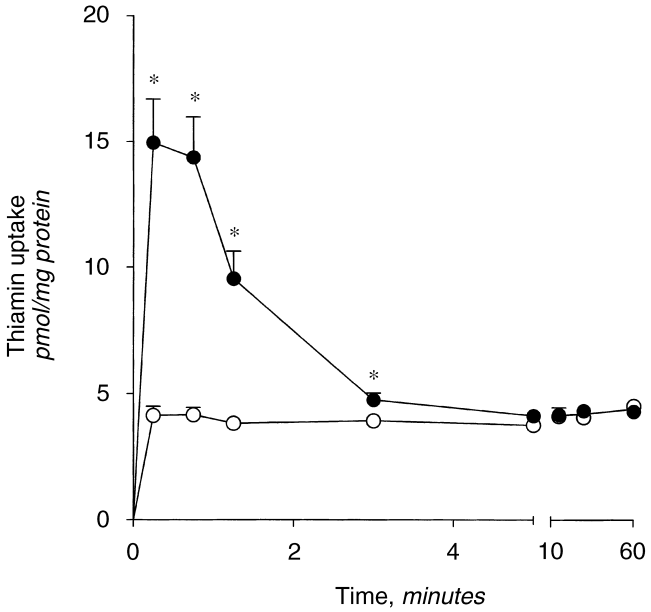

Fig. 5. Effect of FCCP [carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone] on H^+ gradient-dependent thiamin uptake by rat renal brush border membrane vesicles. Vesicles suspended in a medium containing (mmol/L) 200 D-mannitol, 2 $MgSO_4$, 50 KCl, 10 Mes-Tris, pH 6, were preincubated as described in Figure 1 with either FCCP (○; 60 μmol/L, final concentration, +FCCP) or an equivalent volume of ethanol (●; 0.4%, final concentration, −FCCP). Ten microliters of preincubated vesicles were incubated at 25°C with 90 μL of a solution containing 0.25 μmol/L [^{3}H]-thiamin, 200 mmol/L D-mannitol, 2 mmol/L $MgSO_4$, 50 mmol/L KCl, and 10 mmol/L Tris-HEPES, pH 7.5. The number of experiments for each symbol is the same as in Figure 1. *$P \leq 0.05$ vs. +FCCP.

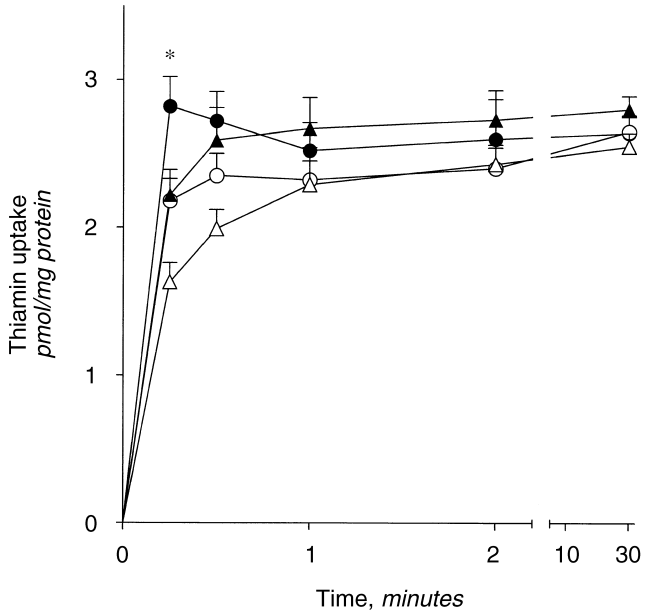

Fig. 6. Effect of transmembrane potential on thiamin uptake by rat renal brush border membrane vesicles. Vesicles were preincubated as described in Figure 1, in a medium containing (mmol/L) 2 $MgSO_4$, 20 Tris-HEPES, pH 7.5, 140 KI (solid symbols), or 140 KCl (open symbols). Ten microliters of preincubated vesicles were incubated at 25°C with 90 μL of a solution containing: 0.25 μmol/L [^{3}H]-thiamin, 2 mmol/L $MgSO_4$, 20 mmol/L Tris-HEPES, pH 7.5, 140 mmol/L KI (triangles), or 140 mmol/L KCl (circles). The number of experiments for each symbol is the same as in Figure 1. *$P < 0.05$ vs. controls (equilibrium-anion conditions) and inside negative potential condition.

vesicular potential difference with the valinomycin-KCl method [26]. BBMVs were pre-equilibrated in a solution containing 100 mmol/L KCl and the K^+ ionophore valinomycin. Thiamin uptakes were measured in transport buffers containing either 100 mmol/L KCl (initial intravesicular electrical potential = 0) or 10 mmol/L KCl and 280 mmol/L D-mannitol (initial intravesicular potential electronegative). Under both conditions, thiamin uptake was similar (Fig. 7). The effectiveness of the procedure in generating an inside negative potential difference was shown by the greater overshoot of the Na^+-dependent D-glucose uptake in the presence of valinomycin compared with its absence (Fig. 8).

Influence of the pH gradient on thiamin translocation and binding

Since at equilibrium thiamin was 53% membrane bound, in preliminary experiments, the time course of thiamin translocation and binding in the presence and the absence of an initial outwardly directed H^+ gradient (pH_{in} 6:pH_{out} 7.5 and pH_{in} = pH_{out} = 7.5, respectively) were evaluated according to the method of Bhandari, Joshi and McMartin [27], as described by Laforenza, Orsenigo and Rindi [5]. Briefly, vitamin uptake was measured in incubation media with increased osmolarities in the two previously mentioned conditions. For each incubation time, the binding component was calculated under iso-osmotic conditions from the ordinate intercepts of the straight lines resulting from the plots of thiamin total uptake values against the reciprocal of medium osmolarities. Translocation was the difference between total uptake and binding values. In the presence of the H^+ gradient, the amount of thiamin translocated up to one minute of incubation time was significantly higher than the amount bound and was constantly over 80% of the total thiamin taken up (Fig. 9). In the absence of gradient, the percentage of thiamin translocated was about 50% at all of the incubation times. It must be emphasized that in the presence of gradient, at a four-second incubation time, as used for the concentration curve (Fig. 10), binding was virtually negligible compared with translocation. At equilibrium (15 to 30 min), translocation and binding were quantitatively similar.

The H^+ gradient and kinetics of thiamin uptake

The effect of an outwardly-directed H^+ gradient on thiamin uptake was examined by comparing the concentration curves obtained at a four-second incubation time under pH gradient (pH_{in} 6:pH_{out} 7.5) and at pH equilib-

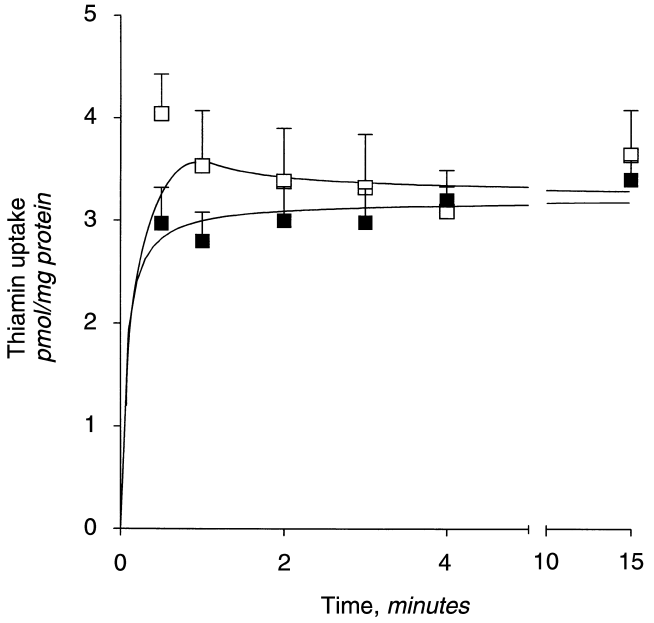

Fig. 7. Effect of transmembrane potential on thiamin uptake by rat renal brush border membrane vesicles. Vesicles were preincubated as described in Figure 1 in a medium containing (mmol/L) 100 KCl, 100 D-mannitol, 2 $MgSO_4$, 10 Tris-HEPES, pH 7.5, with either valinomycin (■; 10 μg/mg protein, final concentration) or an equivalent volume of ethanol (□; 0.1%, final concentration). Ten microliters of preincubated vesicles were incubated at 25°C with 90 μL of a solution containing 0.25 μmol/L [^{3}H]-thiamin, 10 mmol/L KCl, 280 mmol/L D-mannitol, 2 mmol/L $MgSO_4$, 10 mmol/L Tris-HEPES, pH 7.5. Number of experiments for each symbol is the same as in Figure 1.

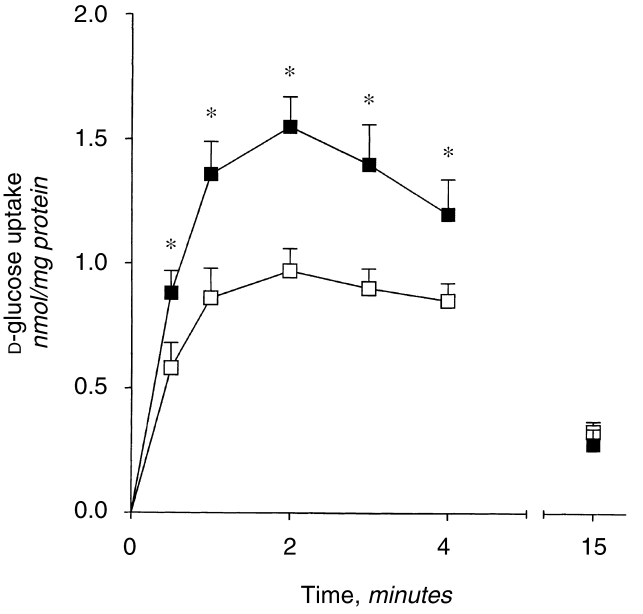

Fig. 8. Effect of transmembrane potential on glucose uptake by rat renal brush border membrane vesicles. Vesicles were preincubated as described in Figure 1 in a medium containing (mmol/L) 100 KCl, 100 D-mannitol, 2 $MgSO_4$, 10 Tris-HEPES, pH 7.5, with either valinomycin (■; 10 μg/mg protein, final concentration) or an equivalent volume of ethanol (□; 0.1%, final concentration). Ten microliters of preincubated vesicles were then incubated at 25°C with 90 μL of a solution containing 80 μmol/L [U-^{14}C]-D-glucose, 10 mmol/L KCl, 90 mmol/L NaCl, 100 mmol/L D-mannitol, 2 mmol/L $MgSO_4$, and 10 mmol/L Tris-HEPES, pH 7.5. Number of experiments for each symbol is the same as in Figure 1. *$P \leq 0.05$ vs. ethanol.

rium ($pH_{in} = pH_{out} = 7.5$). In both cases, a biphasic course of total thiamin uptake was observed, which was nonlinear at low concentrations and linear at higher concentrations (Fig. 10). No correction was introduced for binding, since this was virtually negligible (Fig. 9). The best fit of the curves, calculated by computerized least-square regression (GraphPad Prism 2.01; GraphPad for Windows 95 Software, San Diego, CA, USA, 1996), could be resolved graphically [28] into two components: a linear component, expressed as a nonsaturable mechanism, and a hyperbolic component, expressed as a saturable mechanism with Michaelis-Menten kinetics. The values of the passive permeability coefficients (K_D), calculated as the slope of the linear component of the curves, were (means ± SEM of 4 determinations) 0.62 ± 0.09 at pH equilibrium, and 1.07 ± 0.28 μL/mg protein · 4 s at pH gradient. The difference was not statistically significant. The apparent kinetic constants of the saturable component were K_m 0.29 ± 0.14 μmol/L and J_{max} 4.69 ± 1.17 pmol/mg protein · 4 s in the absence of the pH gradient, and 3.86 ± 0.88 μmol/L and 56.44 ± 6.79 pmol/mg protein · 4 s in the presence of the pH gradient, respectively. The differences were statistically significant ($P < 0.007$ and $P < 0.0005$, respectively, Student's *t*-test), being higher in the presence of the outwardly-directed H^+ gradient.

Effect of different organic cations

In the presence of the pH gradient 6_{in}:7.5_{out}, unlabeled thiamin, different thiamin analogues or derivatives, and organic cations (25 μmol/L, final concentration) were initially added to the incubation medium reported in Figure 10, containing 0.25 μmol/L [^{3}H]-thiamin. Saturable thiamin transport was evaluated by subtracting the nonsaturable component measured at 0°C from the total transport measured at 25°C. The unlabeled thiamin and thiamin analogues and derivatives tested inhibited the saturable component of thiamin uptake (Table 1), with 4′-oxythiamin and thiochrome being only slightly active. Among the exogenous organic cations used, organic cations active on intestinal thiamin/H^+, as well as on renal guanidine/H^+ and Na^+/H^+ antiporters (Table 1, group C), were found to be strong inhibitors and likewise some typical substrates of renal organic cation/H^+ antiport (Table 1, group A). In contrast, other substrates of renal organic cation/H^+ antiport, guanidine (Table 1, group A) and endogenous inhibitors of intestinal guanidine/H^+ antiport (Table 1, group B) were all virtually ineffective.

Countertransport experiments were performed with organic cations active on thiamin/H^+ antiport to shed further light on the mechanism of thiamin transport in

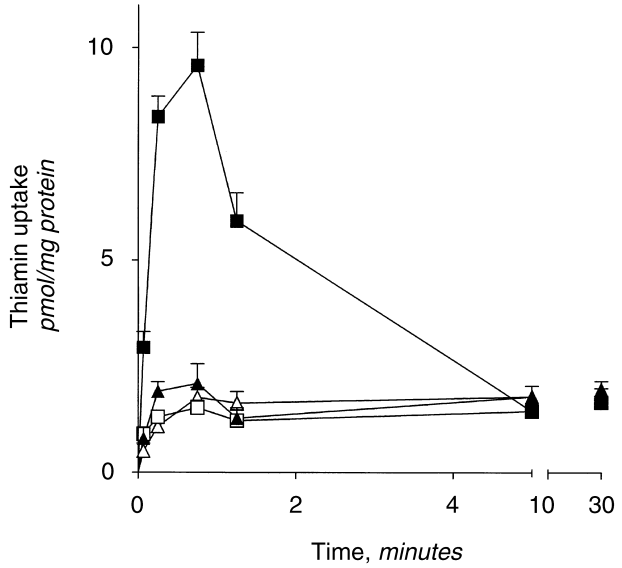

Fig. 9. Time course of thiamin binding and translocation by rat renal brush border membrane vesicles in the presence of a pH gradient (pH_{in} 6:pH_{out} 7.5) and in its absence (pH_{in} = pH_{out} = 7.5). Vesicles were preincubated as described in Figure 1 in a medium containing (mmol/L) 100, 300, or 700 D-mannitol; 50 KCl; 2 $MgSO_4$; 10 Mes-Tris, pH 6 (filled symbols); or 10 Tris-HEPES, pH 7.5 (open symbols), and were incubated at 25°C in a medium containing 0.25 μmol/L [^{3}H]-thiamin, 50 mmol/L KCl, 2 mmol/L $MgSO_4$, 10 mmol/L Tris-HEPES, pH 7.5, and 100, 300, or 700 mmol/L D-mannitol. At each time, the binding (triangles) was determined as in Figure 3 (discussed in the **Results** section). Translocation (squares) was the difference between total uptake and binding (triangles) values as calculated under iso-osmotic conditions (Fig. 3 and **Results** section). The number of experiments for each symbol is the same as in Figure 1.

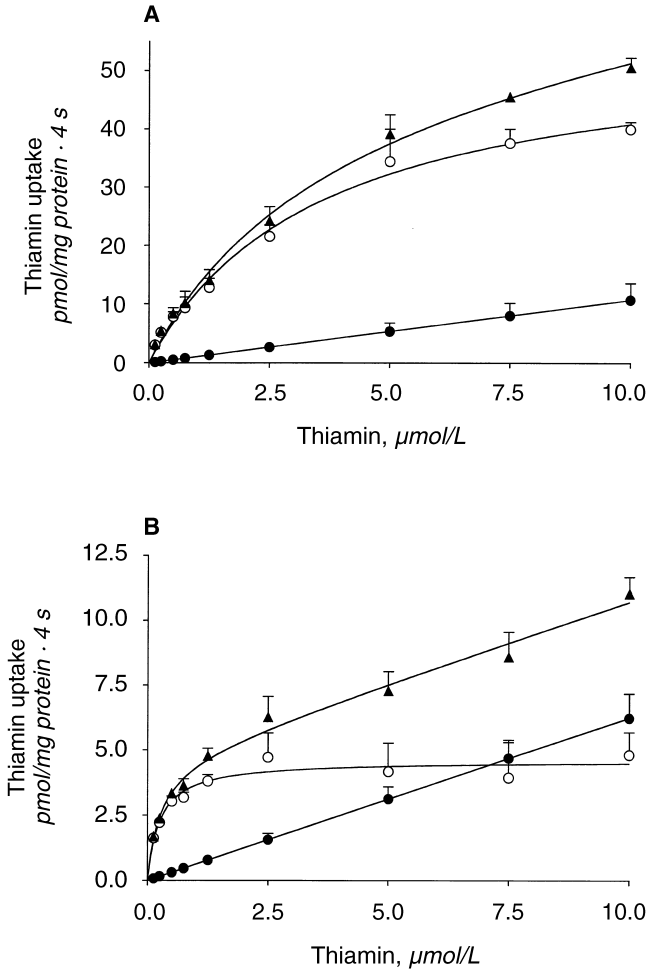

Fig. 10. Effect of increasing thiamin concentrations on the initial rate of thiamin uptake by rat renal brush border membrane vesicles in the presence of a pH gradient (pH_{in} 6:pH_{out} 7.5; *A*) and in its absence (pH_{in} = pH_{out} = 7.5; *B*). Thiamin uptake at 25°C was measured in vesicles, preincubated as described in Figure 1 in a medium containing (mmol/L) 50 KCl, 200 D-mannitol, 2 $MgSO_4$, 10 Mes-Tris, pH 6, or 10 Tris-HEPES, pH 7.5, after four seconds of incubation with different [^{3}H]-thiamin concentrations, 50 mmol/L KCl, 200 mmol/L D-mannitol, 2 mmol/L $MgSO_4$, 10 mmol/L Tris-HEPES, pH 7.5. The number of experiments for each symbol is the same as in Figure 1. The cumulative uptake curves and the values of the apparent K_m and J_{max} constants of the saturable components were obtained by fitting the experimental points with computerized least-squares regression. The saturable and nonsaturable components were obtained graphically [28] from the cumulative curves. Symbols are: (▲) cumulative; (○) saturable; (●) nonsaturable.

rat renal BBMVs. In the absence of a pH gradient, the uptake of 1 μmol/L [^{3}H]-thiamin at 15 seconds significantly increased in BBMVs preloaded with 200 μmol/L amiloride or cimetidine, whereas it remained unmodified in BBMV preloaded with 200 μmol/L harmaline (Table 2). The presence of 200 μmol/L clonidine, imipramine, or quinine inside the vesicles significantly inhibited the initial thiamin uptake (Table 2).

The concentration response relationships of exogenous cations (Table 1, Group C) active on pH gradient-dependent [^{3}H]-thiamin uptake in BBMVs were examined by Dixon plots [29] and Cornish-Bowden plot [30] using two substrate concentrations (0.5 and 1 μmol/L) at a four-second incubation time. Amiloride and cimetidine, which were found to trans-stimulate thiamin uptake, were competitive inhibitors with low K_i values (Table 2). Clonidine, imipramine, and quinine inhibited the saturable component of thiamin/H^+ antiport in a mixed fashion. Moreover, harmaline apparently showed an uncompetitive inhibition mechanism on thiamin/H^+ antiport (Table 2).

Stoichiometry of thiamin/H^+ antiport

This was evaluated by applying the static-head test as described by Wright and Wunz [25] for renal tetraethylammonium/H^+ antiport. Briefly, the native KCl conductance of rat renal BBMVs clamps the potential difference (PD) at 0 mV when 150 mmol/L KCl [31] was present in both intravesicular and extravesicular solutions [25]. Under this condition, by varying the transmembrane gradients of H^+ and thiamin and measuring the effect on net thiamin flux, it is possible to calculate the appropriate value of the exchange ratio. BBMVs were preloaded with 1 μmol/L [^{3}H]-thiamin in a buffer of pH 6.0 containing 150 mmol/L KCl (Fig. 11). The vesicles were then

Table 1. Effect of unlabeled thiamin, thiamin analogues and derivatives, and some organic cations on the saturable component of the thiamin/H^+ antiport in rat renal brush border membrane vesicles

Group	Compounds tested	Thiamin uptake *pmol/mg protein · 4 s* (mean ± SEM)[a]	Percent activity
Control	None	4.25 ± 0.32	100
	Thiamin analogues and derivatives		
	Thiochrome	3.29 ± 0.43	77
	4′-oxythiamin	3.22 ± 0.42	75
	Amprolium	1.54 ± 0.39[b]	36
	Pyrithiamin	1.54 ± 0.39[b]	36
	Unlabeled thiamin	0.62 ± 0.18[b]	15
	Organic cations		
Group A	Creatinine	4.28 ± 0.39	100
	Choline	4.32 ± 0.65	100
	Guanidine	4.25 ± 0.44	100
	Tetraethylammonium	3.92 ± 0.4	92
	Cimetidine	0.58 ± 0.14[b]	14
	Quinine	0.58 ± 0.23[b]	14
Group B	Histamine	4.85 ± 0.16	100
	Spermidine	4.15 ± 0.5	98
	Serotonin	3.78 ± 0.46	89
Group C	Clonidine	1.06 ± 0.29[b]	25
	Imipramine	0.86 ± 0.24[b]	20
	Amiloride	0.67 ± 0.19[b]	15
	Harmaline	0.07 ± 0.02[b]	2

The [^{3}H]-thiamin concentration was 0.25 μmol/L; incubation time was 4 seconds and the H^+ gradient, pH_{in} 6:pH_{out} 7.5. For incubation media and experimental conditions, see the legend of Figure 10. Organic cations, unlabeled thiamin, thiamin analogues and derivatives were added to the incubation medium at an initial 25 μmol/L concentration. Groups are defined as: Group A, substrates of renal organic cation/H^+ antiport [39]; Group B, endogenous inhibitors of intestinal guanidine/H^+ antiport [40]; Group C, exogenous inhibitors of the antiporters, intestinal thiamin/H^+ [5], renal guanidine/H^+ and/or renal Na^+/H^+ [39, 42].

[a]Means of at least triplicate determinations for each of five different preparations, each from 5–6 rats

[b]$P < 0.05$ vs. control

diluted 10-fold with transport solutions buffered to pH values 6.0, 6.5, 7.0, or 7.5. Under these conditions, at 0 time incubation a ten-fold outwardly directed chemical gradient of thiamin developed, favoring both passive and carrier-mediated efflux of thiamin from the vesicles. Thiamin efflux could be countered by an outwardly directed H^+ gradient of appropriate magnitude, resulting in a steady-state condition for thiamin/H^+ exchange (the static-head condition). If the exchange thiamin/H^+ is one to one, the outwardly directed thiamin gradient would be balanced when the external pH was 7.0, whereas a greater external pH should cause a net intravesicular accumulation of thiamin. If the exchange is $2H^+$ per one T, the static-head should exist at an external pH of 6.5, whereas at a greater external pH, a net accumulation of thiamin should result. In Figure 11, the static-head was evident at an external pH of 7.0, and a net accumulation of thiamin was only noted when external pH was raised to 7.5. Therefore, the exchange thiamin/H^+ can be rightly described as a one to one exchange.

DISCUSSION

The functional efficiency of our renal BBMV preparations, evaluated as the time course of D-glucose uptake (Fig. 1), and their purity, evaluated as the enrichment of alkaline phosphatase and K^+-activated phosphatase activities, were similar to those reported in the literature and thus were considered suitable for transport studies.

The time course profile of thiamin uptake by renal efficiently transporting BBMVs was virtually identical in both the presence and the absence of Na^+ (Fig. 2). Thus, as for the enterocyte [4], Na^+ was not necessary for thiamin entry into the renal epithelial cell. Of the thiamin taken up by BBMVs, only 47% was translocated into the intravesicular space with the remainder being membrane bound (Fig. 3), a finding similar to that observed in intestinal BBMVs [5].

At concentrations of [^{3}H]-thiamin in the incubation medium below approximately 1.25 μmol/L, the vesicular uptake of thiamin displayed the features of a saturable process of the Michaelis-Menten type. Since physiological plasma concentrations do not usually exceed 0.24 μmol/L in the rat [12, 13], the renal reabsorption of thiamin, at least in the earliest proximal convoluted tubule where pH is 7.4, appears to be mostly accounted for by a saturable mechanism.

The presence of a H^+ gradient across the vesicle membrane enhanced [^{3}H]-thiamin uptake only when the direction of the gradient was from inside to outside, that is, $pH_{in} < pH_{out}$ (Figs. 4 and 5), suggesting that the entry of thiamin into the renal epithelial cell is associated with a countertransport of H^+ (thiamin/H^+ antiport). A similar antiportal thiamin/H^+ exchange has recently been

Table 2. Trans-effect, inhibition type and apparent inhibitory constant values (K_i) of some exogenous organic cations on the saturable component of the thiamin/H^+ exchanger in rat renal brush border membrane vesicles

Organic cation	Trans-effect[a] *percent activity*[d]	Inhibition type[b]	
		Type	K_i *μmol/L*[c]
None (controls)	100		
Amiloride	148 ± 9.5[e]	Competitive	13.6
Cimetidine	215 ± 64[e]	Competitive	12.2
Clonidine	38 ± 17[e]	Mixed	33
Imipramine	61 ± 12[e]	Mixed	45
Quinine	26 ± 9[e]	Mixed	31
Harmaline	99 ± 0.05	Uncompetitive	19

[a]Vesicles were preloaded (Fig. 5) with 200 μmol/L organic cation, or without cation (controls), in a medium containing 200 mmol/L D-mannitol, 50 mmol/L KCl, 2 mmol/L $MgSO_4$, 10 mmol/L Tris-Hepes, pH 7.5, with FCCP (60 μmol/L, final concentration) and valinomycin (10 μg/mg protein, final concentration). Ten microliters of preloaded vesicles were incubated at 25°C for 15 seconds with 390 μL of the above-mentioned medium containing 1 μmol/L [^{3}H]-thiamin. The incubation medium of the controls also contained 5 μmol/L organic cation

[b]Inhibition type was evaluated following Dixon [29] by using two thiamin concentrations (0.5 and 1 μmol/L) with increasing concentrations of inhibitor (10, 25, 50, 100 μmol/L) at 4 seconds of incubation. For the experimental conditions and medium compositions see the legend in Fig. 10

[c]K_i: apparent inhibitory constant values were determined by using Dixon plots [29] for competitive and mixed inhibition, and Cornish-Bowden [30] plots for uncompetitive inhibition (see Fig. 12)

[d]Means ± SEM were calculated of at least triplicate determinations for each of five different preparations, each from 4–5 rats

[e]$P < 0.05$ vs. controls before transformation of data into percent activity

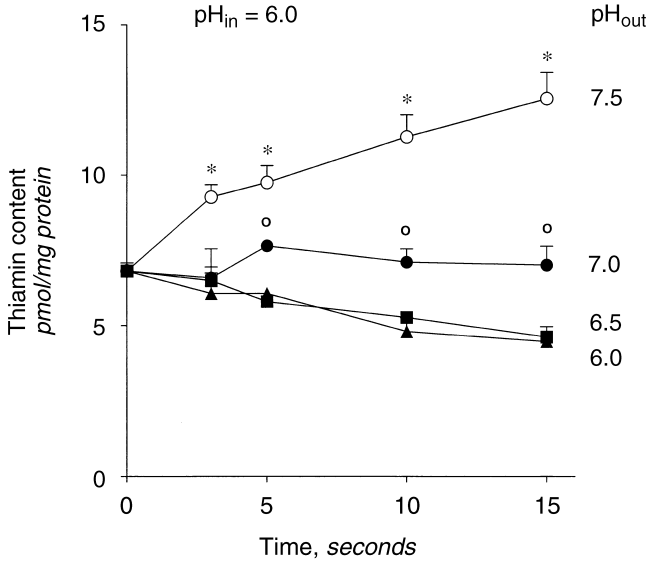

Fig. 11. Effect of opposed thiamin and H^+ gradients on net flux of thiamin in rat renal brush border membrane vesicles. Vesicles were preincubated as described in Figure 1 in a medium containing (mmol/L) 50 KCl, 200 D-mannitol, 2 $MgSO_4$, 10 Mes-Tris, pH 6, and 1 μmol/L [^{3}H]-thiamin and were diluted 10-fold into solutions containing (mmol/L) 50 KCl, 200 D-mannitol, 2 $MgSO_4$, 10 Mes-Tris, pH 6, or 10 Tris-HEPES, pH 6.5, 7, or 7.5. The time course of thiamin content (0 to 15 s) was evaluated at 25°C. Number of experiments for each symbol is the same as in Figure 1. *$P \leq 0.05$ vs. time zero; ≤ 0.05 vs. pH$_{out}$ 6 and 6.5.

shown in rat liver sinusoidal membrane vesicles, where it was different from the organic cation/H^+ and Na^+/H^+ antiports [32], and in rat intestinal [5] and human placental epithelium [33] BBMVs.

As a quaternary ammonium compound, thiamin is positively charged (monovalent cation) at pH 7.5 [34]. However, its uptake was not influenced by changes in membrane electrical potential (Figs. 6 and 7), particularly by an inside negative potential theoretically favoring cation thiamin transport across the membrane. This suggests that thiamin transport by renal BBMVs is an electroneutral process as in intestinal BBMVs [5]. Other electroneutral organic cation/H^+ antiport systems have been shown in renal BBMVs [25, 35, 36]. However, transport of thiamin in BBMVs was stimulated by electrical positivity of the vesicle interior (Fig. 6). According to Wright and Wunz, this PD sensitivity of transport may facilitate the transepithelial secretory flux of organic cations in vivo, where the flow is from the cytoplasm to tubular lumen [25].

At least theoretically, the pH gradient 6_{in}:7.5_{out}, which enhanced the transport of thiamin into BBMVs, could also influence membrane binding. Indeed, its influence depended on the incubation time (Fig. 9), being negligible at short incubation times (up to 30 s), when the pH gradient was virtually intact and translocation accounted for about 83% of thiamin taken up. At long incubation times, when the pH gradient was dissipated, about 55% was membrane bound (Fig. 3).

The uptake of thiamin by exchange with H^+ showed a well-defined saturable component, in which the apparent kinetic constants were much higher than those calculated in the absence of H^+ exchange. At a pH gradient of 1.5 unit (pH$_{in}$ 6:pH$_{out}$ 7.5), both K_m and J_{max} values increased about 12 times (Fig. 10). Because membrane passive permeability was virtually unaffected, as the unaltered values of K_D coefficient indicated, the conclusion can be drawn that the H^+ gradient energized thiamin transport, increasing the maximal flux of the vitamin, while reducing its affinity for the transport mechanism. A similar result was recently found in small intestinal BBMVs of rats, although it was quantitatively lower and with a 2.5 unit pH gradient [5]. The twice higher increment of J_{max} value in renal compared with intestinal vesicles could be reasonably ascribed to the different density of transport sites in renal membranes.

Present findings indicate that the saturable component of thiamin transport into renal BBMVs, like that in the small intestine [5], is a Na^+- and electrical-independent uphill process (Fig. 4), using the energy supplied by an outward H^+ gradient (Fig. 5) and a stoichiometric thiamin/H^+ exchange of 1:1 (Fig. 11).

As to the specificity of thiamin/H^+ antiporter, it appears to be different from either the polyspecific H^+/cation antiporter used by tetraethylammonium, choline, N′-methylnicotinamide, β-lactam antibiotics (system i), or the more

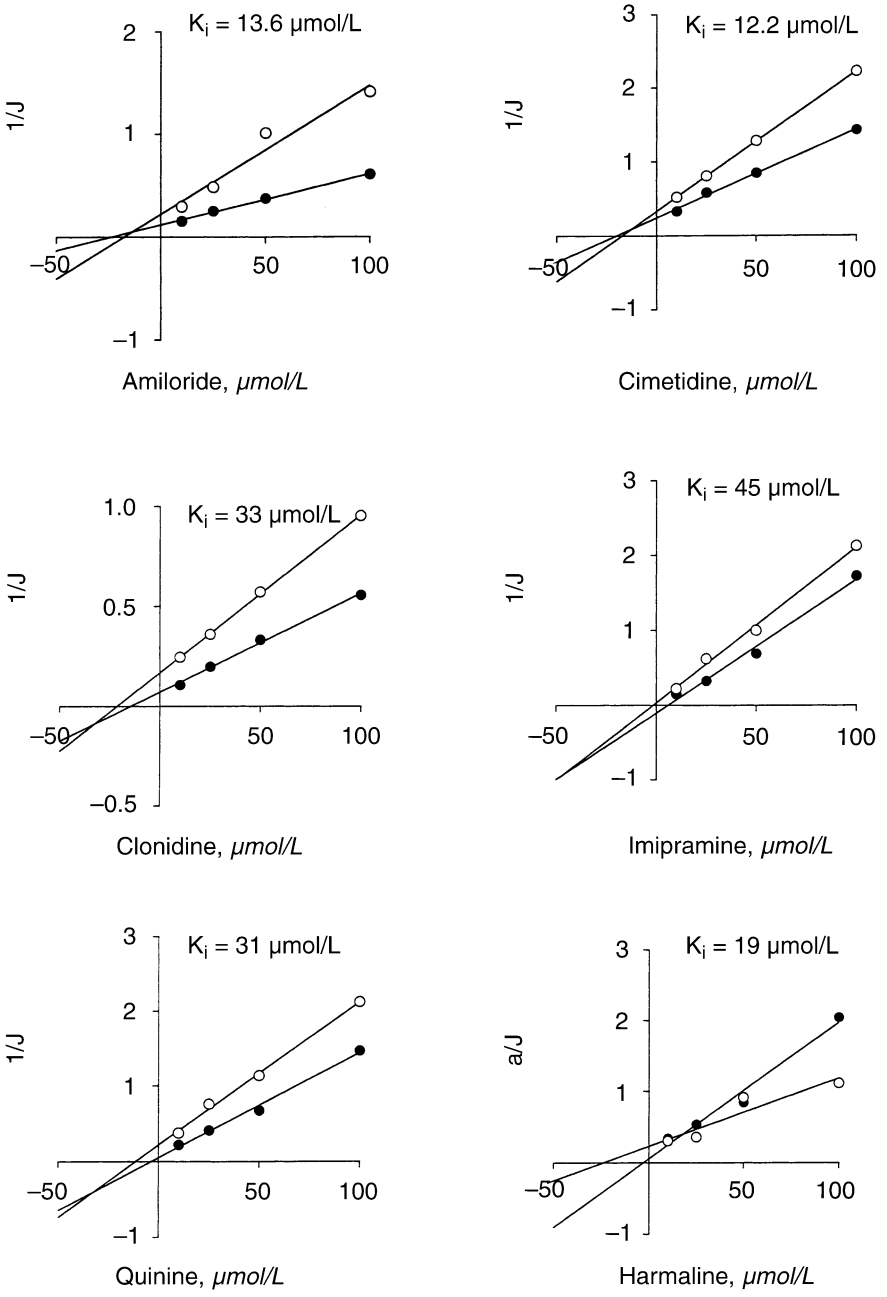

Fig. 12. Calculation of the inhibitory constant (K_i) for the inhibition thiamin uptake in rat renal brush border membrane vesicles caused by some exogenous organic cations. K_i values for amiloride, cimetidine, clonidine, imipramine and quinine were calculated according to Dixon [29], while the K_i value for harmaline was calculated following Cornish-Bowden [30]. Uptake at 4 seconds was measured in the presence of a pH gradient (pH_{in} 6:pH_{out} 7.5). Refer to Table 2 for the number of experiments for each symbol, the incubation media and experimental conditions. J represents the uptake values expressed as pmol/mg protein · 4 s; a is the thiamin concentration in µmol/L.

specific H^+/cation antiporter-transporting guanidine (system ii), both present in renal BBMVs and responsible for organic cation reabsorption and/or secretion [37–40]. As shown in Tables 1 and 2 and Fig. 12, typical substrates of these antiporters can use or inhibit thiamin/H^+ antiport [quinine and cimetidine for system (i), and clonidine, amiloride, imipramine, and harmaline for system (ii)], whereas others cannot inhibit thiamin uptake [tetraethylammonium, choline, creatinine for system (i) and guanidine for system (ii)]. However, thiamin/H^+ renal exchanger was very similar to that found in intestinal BBMVs [5], being inhibited by the same inhibitors, including unlabeled thiamin, thiamin analogues, and derivatives (Table 1). Moreover, it appears to be strictly related to the Na^+/H^+ exchanger, as it is primarily responsible for the acidification of tubular fluid in the renal proximal tubule [41], being inhibited by some of its exogenous inhibitors [42], including diethylpyrocarbonate (abstract; Verri et al, *Pflügers Arch* 435: R38, 1998), which is a histidine-specific reagent irreversibly blocking the renal BBMV Na^+/H^+ exchanger [43].

At physiological plasma concentrations, thiamin is usually reabsorbed by the rat kidney, since its plasma clearance is lower than that of inulin: 0.6 mL/min · kg

body weight (Patrini and Laforenza, unpublished results) versus 5.9 mL/min · kg body weight [44]. According to our study results, thiamin reabsorption takes place through the thiamin/H^+ exchange mechanism in the early proximal convoluted tubule, the renal structure with the highest acidification rate, predominantly through the luminal antiporter Na^+/H^+ [45]. The crossing of thiamin through the luminal plasma membrane is facilitated by the low thiamin intracellular concentration maintained by its rapid conversion to thiamin pyrophosphate [46]. This metabolic trap could produce an effective means by which thiamin could be cleared from the tubular fluid. The successive exit of thiamin through the basolateral membrane of the tubular cells into the plasma could be achieved by its active basolateral extrusion by Na^+,K^+-ATPase, as has been shown for small intestinal enterocytes [6].

As mentioned previously in this article, thiamin can be also secreted into the renal tubule [11]. If thiamin crossing through the brush border membrane is determined by the thiamin/H^+ exchange mechanism, and following Aronson [47], the direction of thiamin flux depends on whether the electrochemical proton gradient ($\Delta\eta_{H+}$) across the membrane is higher or lower than the thiamin gradient ($\Delta\eta_{T+}$). Both gradients can be calculated according to Yoshitomi and Frömter [48]:

$$\Delta\eta_{H+} = RT \cdot \ln[S]_{in}/[S]_{out} + F \cdot \Delta\phi \cdot Z_S$$

where S is the cation transported, $\Delta\phi$ is the electrical potential difference across the luminal cell membrane (75 mV), and R, T, F, Z have their usual meanings.

In the early tract of the proximal convoluted tubule, where $pH_{in} = 7.17$ [48] and $pH_{out} = 7.4$, $\Delta\eta_{H+}$ corresponds to 8.68 kJ/mol and is directed from cell to lumen. This is the same tract where, according to Patrini and Laforenza (unpublished results) $[thiamin]_{in} = 0.92$ and $[thiamin]_{out} = 0.69$ μmol/L; thus, $\Delta\eta_{T+}$ corresponds to 7.97 kJ/mol, being directed from cell to lumen. It must be emphasized, however, that the value of $[thiamin]_{out}$ indicated is the sum of plasmatic thiamin plus thiamin monophosphate, since thiamin phosphates of ultrafiltrate are mainly converted to thiamin by membrane phosphatases of the renal tubule [49]. Under this assumption, $\Delta\eta_{T+}$ is lower than $\Delta\eta_{H+}$, and thiamin can be reabsorbed.

It can be predicted that thiamin/H^+ antiport will promote a net secretion of thiamin in the lumen, when $\Delta\eta_{H+}$ inverts its direction as a consequence of an ultrafiltrate pH lower than 7.17 (distal convoluted tubule), and when plasma and intracellular concentrations of thiamin become higher as a consequence of the administration of the vitamin in pharmacological doses. In this condition, it is conceivable that thiamin could cross basolateral membrane not through Na^+,K^+-ATPase intervention, which works in the opposing direction, but probably by the polyspecific transporter OCT1 [17] or by passive diffusion, its transepithelial flux being facilitated by the inside electropositivity (Fig. 6), as has been suggested for the secretion of organic cation tetraethylammonium across the luminal membrane [25].

The experimental data presented here provide strong evidence for the presence of a thiamin/H^+ antiport mechanism, which seems to be rather specific for thiamin in rat renal brush border membranes. This conclusion is supported by the following observations: (*1*) Thiamin uptake by renal BBMVs is stimulated several-fold by an outwardly directed H^+ gradient. (*2*) In the presence of the H^+ gradient, a transient accumulation of thiamin inside the vesicles against a concentration gradient occurs. (*3*) The stimulation of thiamin uptake caused by the H^+ gradient is not due to an inside negative H^+-diffusion potential. (*4*) Different thiamin analogues, and some typical organic cations, strongly compete with thiamin for the uptake process. (*5*) The thiamin/H^+ antiport is strictly related to the activity of the Na^+/H^+ antiporter and displays a stoichiometric thiamin/H^+ ratio of 1:1.

ACKNOWLEDGMENTS

This study was supported by the grant n. 95.02410.CT04 from Consiglio Nazionale delle Ricerche, CNR, Rome, Italy. A preliminary account of a portion of this study was communicated to the XXIII meeting of the Italian Physiological Society (1996).

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REFERENCES

1. Rindi G: Thiamin, in *Present Knowledge in Nutrition*, edited by Ziegler EE, Filer LJ, Washington D.C., IlSI Press, 1996, pp 160–166
2. Rindi G, Ventura U: Thiamine intestinal transport. *Physiol Rev* 52:521–527, 1972
3. Rindi G: Thiamin absorption by small intestine. *Acta Vitaminol Enzymol* 6:47–55, 1984
4. Casirola D, Ferrari G, Gastaldi G, Patrini C, Rindi G: Transport of thiamine in brush border membrane vesicles from rat small intestine. *J Physiol (Lond)* 398:329–339, 1988
5. Laforenza U, Orsenigo MN, Rindi G: A thiamine/H^+ antiport mechanism for thiamine entry into brush border membrane vesicles from rat small intestine. *J Membr Biol* 161:151–162, 1998
6. Laforenza U, Gastaldi G, Rindi G: Thiamine outflow from the enterocyte: A study using basolateral membrane vesicles from rat small intestine. *J Physiol (Lond)* 468:401–412, 1993
7. Ferrari G, Patrini C, Rindi G: Intestinal thiamine transport in rats. *Pflügers Arch* 393:37–41, 1982
8. Rennick BR: The renal tubular excretion of choline and thiamine in the chicken. *J Pharmacol Exp Ther* 122:449–456, 1958
9. Acara M, Rennick B: Regulation of plasma choline by the renal tubule: Bidirectional transport of choline. *Am J Physiol* 225:1123–1128, 1973
10. Acara M, Rennick B: The biphasic effect of organic cations on the excretion of other cations. *J Pharmacol Exp Ther* 199:32–40, 1976
11. Weber W, Nitz M, Looby M: Non-linear kinetics of the thiamine cation in humans: Saturation of non renal clearance and tubular reabsorption. *J Pharmacokinet Biopharm* 18:501–523, 1990
12. Patrini C, Perucca E, Reggiani C, Rindi G: Effects of phenitoin on the in vivo kinetics of thiamine and its phosphoesters in the rat nervous tissues. *Brain Res* 628:179–186, 1993

13. Nauti A, Patrini C, Reggiani C, Merighi A, Bellazzi R, Rindi G: In vivo study of the kinetics of thiamine and its phosphoesters in the deafferented rat cerebellum. *Metab Brain Dis* 12:145–160, 1997
14. Malnic G, Carvalho da Silva A, De Angelis RC, Gomes ZJ: Renal excretion of thiamin by the dog. *Am J Physiol* 198:1274–1278, 1960
15. Rindi G, de Giuseppe L, Sciorelli G: Thiamine monophosphate, a normal constituent of rat plasma. *J Nutr* 94:447–454, 1968
16. Thom JY, Davis RE, Icke GC: Dephosphorylation of thiaminpyrophosphate by fresh human plasma. *Int J Vitam Nutr Res* 55:269–273, 1985
17. Koepsell H: Organic cation transporters in intestine, kidney, liver, and brain. *Annu Rev Physiol* 60:243–266, 1998
18. Randoin L, Causeret J: Constitution d'un régime alimentaire équilibré pour les jeunes rats blancs destinés au dosage des vitamines A et D par les méthodes biologiques. *Bull Soc Hyg Aliment* 35:14–18, 1947
19. Biber J, Stieger B, Haase W, Murer H: High yields preparation for rat kidney brush border membranes: Different behaviour of lysosomal markers. *Biochim Biophys Acta* 647:169–179, 1981
20. Murer H, Ammann E, Biber J, Hopfer U: The surface membrane of the small intestinal epithelial cell. I. Localization of adenyl cyclase. *Biochim Biophys Acta* 433:509–519, 1976
21. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:443–449, 1951
22. Kessler M, Acuto O, Storelli C, Murer H, Semenza G: A modified procedure for the rapid preparation of efficiently transporting vesicles from small intestinal brush border membranes. *Biochim Biophys Acta* 506:136–154, 1978
23. Glantz SA: *Statistica Per Discipline Biomediche: Programma Applicativo (Statistics for Biomedical Disciplines: Applicative Program).* Milano, McGraw-Hill Libri Italia, 1988
24. Aronson PS, Sacktor B: The Na^+ gradient-dependent transport of D-glucose in renal brush border membrane. *J Biol Chem* 250:6032–6039, 1975
25. Wright SH, Wunz TM: Transport of tetraethylammonium by rabbit renal brush border and basolateral membrane vesicles. *Am J Physiol* 253:F1040–F1050, 1987
26. McKinney TD, Kunnemann ME: Procainamide transport in rabbit renal cortical brush-border membrane vesicles. *Am J Physiol* 249:F532–F541, 1985
27. Bhandari SD, Joshi SK, McMartin KE: Folate binding and transport by rat kidney brush-border membrane vesicles. *Biochim Biophys Acta* 937:211–218, 1988
28. Gastaldi G, Casirola D, Ferrari G, Rindi G: Effect of chronic ethanol administration on thiamine transport in microvillous vesicles of rat small intestine. *Alcohol Alcoholism* 24:83–89, 1989
29. Dixon M: The determination of enzyme inhibitor constants. *Biochem J* 55:170–171, 1953
30. Cornish-Bowden A: *Fundamentals of Enzyme Kinetics.* London, Portland Press Ltd., 1995
31. Ives HE, Chen PY, Verkman AS: Mechanism of coupling between Cl^- and OH^- transport in renal brush-border membranes. *Biochim Biophys Acta* 863:91–100, 1986
32. Moseley RH, Vashi PG, Jarose SM, Dickinson CJ, Permoad PA: Thiamine transport by basolateral rat liver plasma membrane vesicles. *Gastroenterology* 103:1056–1063, 1992
33. Grassl SM: Thiamine transport in human placental brush border membrane vesicles. *Biochim Biophys Acta* 1371:213–222, 1998
34. Komai T, Shindo H: Structural specificities for the active transport system of thiamine in rat small intestine. *J Nutr Sci Vitaminol* 20:179–187, 1974
35. Ott RJ, Hui AC, Yuan G, Giacomini KM: Organic cation transport in human renal brush-border membrane vesicles. *Am J Physiol* 261:F443–F451, 1991
36. Ullrich J: Renal transporters for organic anions and organic cations. Structural requirements for substrates. *J Membr Biol* 158:95–107, 1997
37. Koepsell H, Busch A, Gorboulev V, Arndt P: Structural and function of renal organic cation transporters. *News Physiol Sci* 13:11–16, 1998
38. Wright SH, Wunz TM, Wunz TP: A choline transporter in renal brush-border membrane vesicles: Energetics and structural specificity. *J Membr Biol* 126:51–56, 1992
39. Miyamoto Y, Tiruppathi C, Ganapathy V, Leibach FH: Multiple transport systems for organic cations in renal brush border membrane vesicles. *Am J Physiol* 256:F540–F548, 1989
40. Miyamoto Y, Ganapathy V, Leibach FH: Transport of guanidine in rabbit intestinal brush border membrane vesicles. *Am J Physiol* 255:G85–G92, 1988
41. Aronson PS: Mechanisms of active H^+ secretion in the proximal tubule. *Am J Physiol* 245:F647–F659, 1983
42. Kinsella JL, Aronson PS: Properties of the Na^+-H^+ exchanger in renal microvillous membrane vesicles. *Am J Physiol* 238:F461–F469, 1980
43. Grillo FG, Aronson PS: Inactivation of the renal microvillous membrane Na^+-H^+ exchanger by histidine-specific reagents. *J Biol Chem* 261:1120–1125, 1986
44. Altman PL, Dittmer DS: *Biological Data Book* (vol 3, 2nd ed). Bethesda, Federation of American Societies for Experimental Biology, 1974, p 2004
45. Cogan MG, Quan AH: Renal acidification: Integrated tubular responses, in *Handbook of Physiology* (section 8, 3rd ed, vol 1), edited by Windhager EE, Oxford, Oxford University Press, 1992, pp 974–975
46. Mahajan MA, Acara M, Taub M: Uptake and phosphorylation of thiamine in rabbit primary proximal tubule cells and Madin Darby canine kidney cells. II. Effect of ethanol. *J Pharmacol Exp Ther* 268:1316–1320, 1994
47. Aronson PS: Kinetic properties of the plasma membrane Na^+-H^+ exchanger. *Annu Rev Physiol* 47:545–560, 1985
48. Yoshitomi K, Frömter E: Cell pH of rat renal proximal tubule in vivo and the conductive nature of peritubular HCO_3^- (OH^-) exit. *Pflügers Arch* 402:300–305, 1984
49. Malnic G, Rocha Silva M Jr, De Angelis RC, Carvalho da Silva A: Simultaneous excretion of thiamine and thiaminephosphates by the dog. *Am J Physiol* 202:1109–1114, 1962